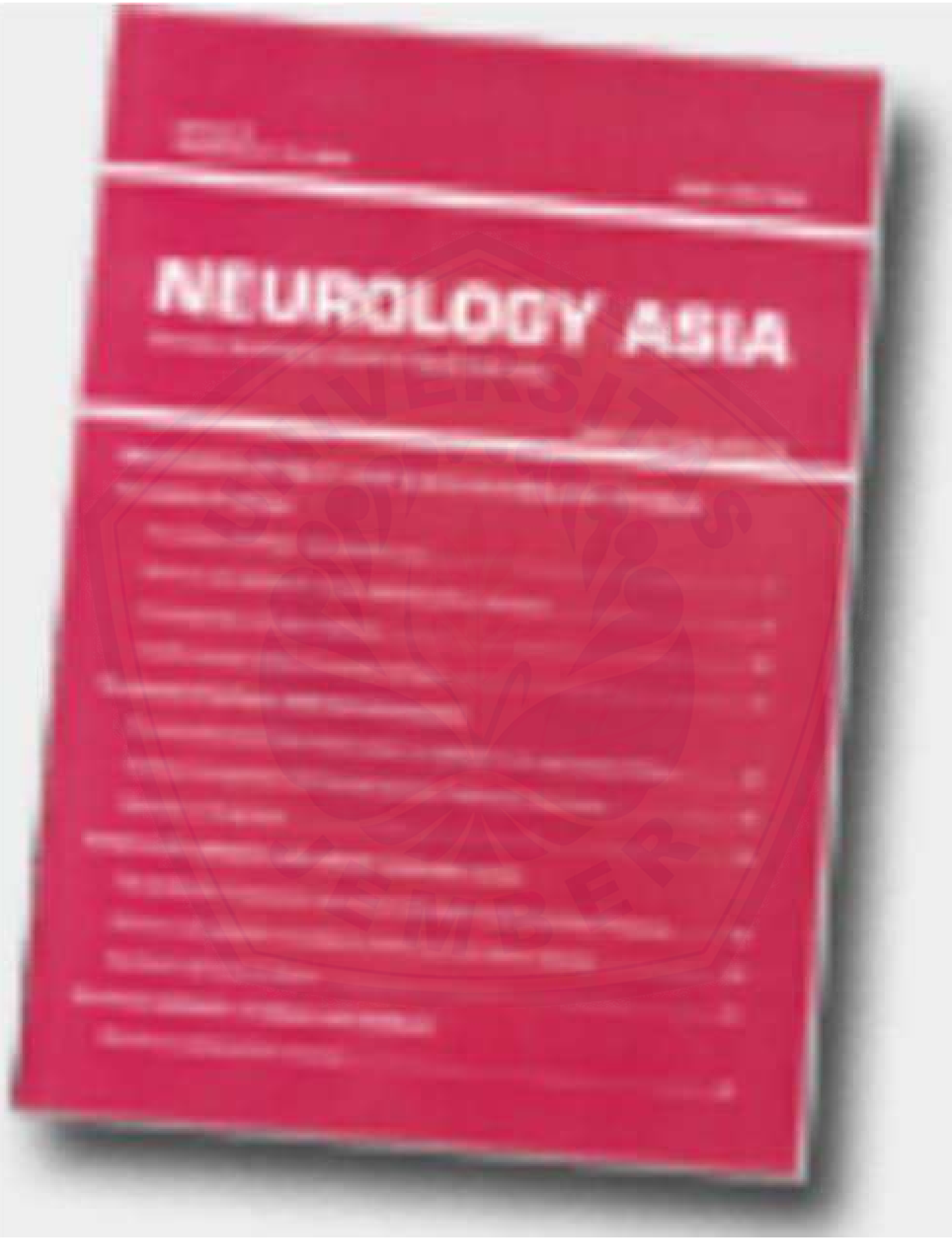

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Neural stem cell secretome injection decreased neuropathic pain expression on subacute spinal cord injury rats model

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Abstract

Background: Neuropathic pain is one of the quality of life problems after spinal cord injury (SCI) that is caused by the lack of neurotrophic agents that modulate the regeneration process. The neural stem cell (NSC)-secretome has the potential as a neurotrophic agent for palliative treatment after SCI. The effect of NSC-secretome is still unproven. The aim of this study is to investigate the effect of NSC-secretome on neuropathic pain. *Methods:* In this experimental study, ten male rats were divided into two groups. The first group did not receive NSC-secretome. The second group was injected with NSC-secretome (30 μL) intrathecally into the injury site**.** The neuropathic pain was measured using a real-time rat grimace scale in the 3rd and 4th weeks after injury. *Result:* The result of the Statistical Package for the Social Sciences (SPSS) independent sample t-test analysis shows a significant difference between a group without and with NSC-secretome administration in the 3rd week ($p<0.001$) and 4th week ($p=0.004$). NSC-secretome group significantly decreased the neuropathic pain compared to the control group.
Conclusion: NSC-secretome injection is able to reduce the neuropathic pain expression on subacute SCI rats model.

Keywords: Spinal cord injury, neural stem cell secretome, neuropathic pain; Sprague Dawley

INTRODUCTION

Spinal cord injury (SCI) is a medical condition when the nerve axon and neuron in the spinal cord is injured with loss of motor and sensory function.[1] The spinal cord in rats has similar morphology to humans, such as the presence of pia mater, arachnoid mater, and dura mater.[2] Pathologically, rats are rather similar to the human spinal cord[3] and have analogous relations with functional, electrophysiological, and morphology.[4] Pain in patients after SCI is one of the symptoms which can reduce the quality of life.[5]

Neuropathic pain (NP) is one of the most prevalent complications of SCI.[6] Ischemic on the spinal cord after SCI induces neuron and glial cell apoptosis, inflammation, and glial scar formation that inhibits regeneration.[7] The cellular process causes NP.[8] It is estimated that 60-69% of patients with SCI suffer from pain.[9] However, conventional therapy only focuses on the stability of injured areas by operation, rehabilitation for loss of function, preventing secondary injury with corticosteroid, anti-thrombotic, and anti-inflammation.[10,11]

Neural stem cell (NSC)-secretome contains a lot of bioactive compounds and other molecule through paracrine effect for regulating the microenvironment that plays a role in maintaining stem cell characteristics, differentiation[12], and cell life sustainability.[13] NSC-secretome also secrete bioactive molecules for immunomodulator, anti-inflammation, and long-lasting tissue regeneration effects.[14] Gao *et al*. proposed that NSC-secretome be given on the third day after SCI intrathecally to mediate the risk of malignant cells or immune cells.[15] The research question of this study is "What effect does NSC-secretome intrathecal injection has on the NP expression in a subacute SCI rat models?". Therefore, the aim of this research was to investigate the NSC-secretome effect of intrathecal injection on the NP expression in a subacute SCI rat model.

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METHOD

Ethics statement

All animals followed the Dentistry Faculty of Jember University guidelines for the use and care of laboratory animals. The research has been qualified by the ethical committee dentistry faculty at Jember University as certified no. 1.443/UN25.8/KEPK/DL/2021.

Groups and study design

The population in this research was white rats strain Sprague dawley. The sample size used Lemeshow with five male rats for each control and treatment group. Experimental animals that had been already included in inclusion criteria were randomized using a random allocation sampling technique with simple random sampling. Animals were randomly divided into two groups: (1) Control group: contusion-compression was performed by yasargil clip but without NSC-secretome injection (n=5); (2) Treatment group: contusion-compression was performed by yasargil clip and administrated NSC-secretome 30 μL by intrathecal injection (n=5).

Rats and the SCI model

Adult male strain Sprague Dawley rats (350-400 g) aging 90 to 120 days were taken from Gadjah Mada University of Medical Sciences. Rats had free access to food and water. The experiment was conducted for 28 days.[16,17] In the experiment, contusion-compression of the spinal cord was performed using stainless steel yasargil clip (length: 7 mm; weight: 65 gr). Anesthetia was achieved on all Sprague dawley rats using the intraperitoneal application of acepromazine (3 mg/kg) and ketamine (40 mg/kg).[18-21] Partial laminectomy was performed to expose thoracal 10 spinal cord. The rostral and caudal spine were clamped by Adson micro forceps to stabilize the vertebrae column. The dura was still intact while the epidural fat was removed. Yasargil clip was placed 1 mm above the spinal cord, and sudden retraction 150 kDyne for a minute, leading to a contusion-compression on T10 spinal cord. The surgical area was irrigated by physiological saline (10 mL). The skin and muscles were sutured in layers. Basso, Beattie, and Bresnahan's (BBB) locomotor rating scale scores was applied after the injury to confirm the establishment of subacute SCI. After the injury, rats received physiological saline (s.c), tolfenamic acid (4 mg/kg s.c), enrofloxacin (10 mg/kg s.c), and gentamicin (0,1%) for three days. The rats were placed under a heating lamp. The rats were given chow *ad libitum* 30-35 grams or 10% of rat's weight and water as much as 30-35 mL or 10% of rat's weight each day. Manual bladder evacuation and intestine palpation were performed by two veterinarians.[22-24]

Functional assessment

Pain after SCI was evaluated using a real-time rat grimace scale (RT-RGS).[25-29] RT-RGS scores were assessed on the 3rd and 4th weeks after surgery. Rat grimace scale consisted of 4 evaluation facial expressions (orbital, nose/cheek, ear, and mustache) with 0, 1, and 2 scores. For this evaluation, rats were put individually and observed in real-time by the observer. RT-RGS was evaluated for 30 seconds from 0s to 15s and 31s to 45s. There were two scores in one cycle. That cycle was evaluated until 9 minutes and there would be 18 scores for each rat. Total scores were averaged for the final score.

Statistical methods

Data were presented as mean ± SEM. We compared data from real-time rat grimace scale evaluations between the two groups. Data were analyzed using the SPSS application program (IBM Statistic 25). Data were performed in the form of ratio data. The normality test used Shapiro Wilk for knowing data distribution ($p>0.05$). Levene's test was used for testing data homogeneity ($p>0.05$). Data were analyzed using an independent sample t-test (test $p<0.005$ was considered statistically significant).

RESULT

Animals treated with NSC-secretome show reduced neuropathic pain expression compared to control

To determine whether the application of NSC-secretome resulted in reduced NP after SCI, RT-RGS was applied.[26,30] Treatment with NSC-secretome led to a significant decrease in NP in groups treated with NSC-secretome compared to control groups. The differences were statistically significant in third and fourth weeks after subacute SCI (n=5; w3: $p<0.001$, w4: $p<0.005$; Figure 1). Three weeks after trauma, animals in the NSC-secretome treatment group had a score of 0.5278 ± 0.0656 (mean ± SEM) while control animals had a score of 1.2528 ± 0.0279 (mean ± SEM). The difference was highly significant with a p-value

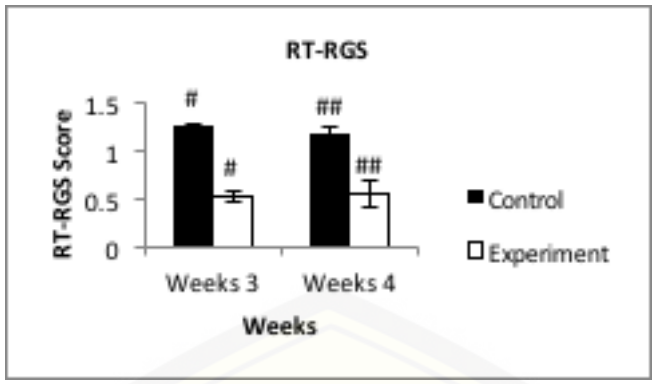

Figure 1. Effect of NSC-secretome on the expression of neuropathic pain using real-time rat grimace scale. The values are shown as mean ± S.E.M. Significant differences between the control group versus the NSC-secretome therapy group (# $p<0.001$ and ## $p<0.005$) were detected.

< 0.001. Four weeks after trauma, animals in the NSC-secretome treatment group had a score of 0.5611± 0.1307 (mean ± SEM) while control animals had a score of 1.1667 ± 0.0697 (mean ± SEM). The difference was highly significant with a p-value < 0.005.

DISCUSSION

This study shows that the average RT-RGS scores three weeks after injury in the treatment group (0.5278 ± 0.1466) is lower than the control group (1.2528 ± 0.0624) while the fourth week after injury also resulted in the average RT-RGS score in the treatment group (0.5611 ± 0.2924) being lower than the control group (1.1667 ± 0.1559). Previous research by Wu *et al*. found that a moderate contusion SCI on adult male mice caused a robust and extended increase of mouse grimace scale up to three weeks after injury.[25] In an in vitro study, Schneider *et al*. also found that experimental models of NP after a contusion SCI at cervical level 5 in adult males resulted in higher RGS scores at week five after injury.[27] Brini *et al*. have demonstrated that intravenous administration of secretome from human adipose-derived stem cells was able to decrease neuropathic pain in diabetic mice which was shown by reversing thermal, mechanical allodynia, thermal hyperalgesia, and re-establishing the correct balance between IL-1β, IL-6, TNF-α, IL-10 in nerves, dorsal root ganglia, and spinal cord of neuropathic mice.[31] In an in an invivo study, Xu *et al*. also demonstrated by using an intrathecal injection of neural stem cells three days after chronic constriction injury could alleviate the neuropathic pain in rats which was shown by attenuating mechanical and thermal hyperalgesia with increasing protein and mRNA levels of a glial cell line-derived neurotrophic factors (GDNF) in the spinal dorsal horn and dorsal root ganglia (DRG).[32] Du *et al*. stated that NSC injection into a rats model of spinal cord injury using a micro-syringe could alleviate neuropathic pain which was shown by suppression of the P2X4R and GFAP receptors-mediated neuropathic pain expression on four weeks after injury.[17] In an in vitro study, Zhang *et al*. demonstrated that microencapsulated neural stem cells (MC-NSCs) in rats alleviate neuropathic pain by decreasing P2 x 4Rs and p-p65, the level of IL-1β, and TNF-α.[33] Table 1 shows the comparison between the previous research by Haider *et al*. and this study.[16]

Secretome express neurotrophic factors secretion that promotes axonal regeneration.[34] It has already been reported that NSC-secretome plays a role in anti-inflammation and immunomodulation after SCI.[14,35] The pain was produced in the spinal cord, DRG, and anterior cingulate cortex with elevated levels of inflammatory cytokines.[36,37] Previous investigations that uses rat models of a contusion on SCI with NSC-secretome also shows an anti-inflammation effect through decreasing cytokines pro-inflammation expression (TNF, IL-1β, IL-6, and IL-12).[35,38-40] NSC-secretome plays a role in anti-inflammation through macrophage pro-inflammation activities inhibition.[30,35,41] It is because activation from the SIRT-1 pathway will deactivate pro-inflammation gene induced and transcription factors such as NF-Kβ and HIF-1α.[14,42] SIRT1 also contributes to the obstruction of pro-inflammation cytokines such as IL-1β.[43]

Besides that, the NSC-secretome also produces TGF-β1, which activates the SIRT-1 pathway and has an anti-inflammation effect.[44,45] Anti-inflammation caused by the NF-kβ antagonist.[14] Those results are in line with previous research

Table 1: The comparison between the previous research by Haider *et al*.[16] and this study

Study	Haider *et al*.[16]	This Study
Study Design	In vivo in rats	In vivo in rats
Type of SCI	*Spinal cord* contusion on T11	*Spinal cord* contusion and compression on T10
Dosage	25 x 106 cells/ml	30 μL
Administration	Intraperitoneal	Intrathecal
Duration	28 days	28 days
Results	Confirmed that the human-derived MNC-secretome attenuates secondary damage after experimental SCI	Confirmed that the human-derived NSC-secretome attenuates neuropathic pain after experimental SCI

that said NSC-secretome plays a role in immunomodulation through bioactive molecule secretion such as prostaglandin E2 (PGE2). PGE2 secretion changes microglia and macrophages from pro-inflammation to anti-inflammation which is showed by IL-1β downregulation.[35] NSC-secretome also secretes TGF-β2 that will decreases pro-inflammation monocyte differentiation.[46] VEGF and TGF-β will promote angiogenesis through PI3K/Akt activation and the MAPK pathway.[47]

The limitation of these studies are the sample size was small, there were no regeneration mechanisms, histopathology and biomarker were not examined. We have confirmed in this study that intrathecal injection of NSC-secretome can decrease NP based on subacute SCI rats model. Further studies on animals as well as clinical trials for humans should be future research directions to pursue.

DISCLOSURE

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Conflict of Interest: None

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